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A novel real time PCR assay using melt curve analysis for ivory identification

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ABSTRACT

Demand for ivory and expansion of human settlements have resulted in a rapid decline in the number of elephants. Enforcement of local and international laws and regulations requires identification of the species from which any ivory, or ivory products, originated. Further geographical assignment of the dead elephant from which the ivory was taken can assist in forensic investigations. In this study, a real-time PCR assay using melt curve analysis was developed and fully validated for forensic use. The presence or absence of three Elephantidae-specific and elephant species-specific melting peaks was used to identify the elephant species. Using 141 blood and ivory samples from the three extant elephant species, the assay demonstrated very high reproducibility and accuracy. The limit of detection was as low as 0.031 ng of input DNA for conventional amplification and 0.002 ng for nested amplification. Both DNA concentrations are typically encountered in forensic casework, especially for degraded samples. No cross-reactivity was observed for non-target species. Evaluation of direct amplification and nested amplification demonstrated the assay's flexibility and capability of analyzing low-template DNA samples and aged samples. Additionally, blind trial testing showed the assay's suitability application in real casework. In conclusion, wildlife forensic laboratories could use this novel, quick, and low-cost assay to help combat the continuing poaching crises leading to the collapse of elephant numbers in the wild.

1. Introduction

Demand for ivory and expansion of human settlements have resulted in a rapid decline in the number of elephants [1]. An effort to prevent their extinction was made in 1989 by the Convention on International Trade in Endangered Species of Flora and Fauna (CITES), which lists all extant elephant species in CITES Appendix I. However, in 1997 and 2000, four African elephant populations (Botswana, Namibia, Zimbabwe, and South Africa) were listed in the Appendix II, which allows regulated trading of their ivory [1]. The change initiated and resulted in further poaching [2]. It is evident by the continuous seizures of ivory and the rise in illegally killed elephants in the past 15 years [1]. In fact, one of the largest-ever ivory destruction (105 tons from over 7000 elephants) was carried out by the Kenyan government early in 2016 [3]. African elephant ivory is often smuggled to black market hotspots such as Thailand and China where trading of the local Asian species is permitted [1]. Therefore, the African elephant ivory is laundered and traded as Asian elephant ivory.

Species identification is a common practice in wildlife crime investigation. Evidence needs to be demonstrated that a seized sample originated from a protected species in order to enforce national legislations. Three elephant species – Asian elephant (*Elephas maximus*), African bush elephant (*Loxodonta africana*), and African forest species (*Loxodonta cyclotis*) – are recognized at present based on morphology and genetics [4–6]. At ivory trade hotspots such as China, Thailand, and Malaysia, species identification is necessary to discriminate local Asian ivory from illegal African ivory, which is illegal. A number of methods have been proposed, including morphology-based macroscopic identification, short-wave near-infrared spectroscopy, and Fourier-transformed Raman spectroscopy [7,8]. These methods are non-destructive and provide both qualitative and quantitative data; however, there are many reasons that prevent them from being

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used in routine forensic investigations. These reasons include the inability to identify the elephant species from which the ivory was taken; expensive equipment; high false positive and negative rates; and lack of robustness for use with highly degraded or processed samples commonly found in crime scenes [7–10].

A molecular approach can overcome these drawbacks and provide greater confidence in species assignment. Molecular markers have been designed based on mitochondrial DNA loci such as cytochrome *b* (cyt *b*) and the control region using either standard or nested polymerase chain reaction (PCR) [11–13]. Significant disadvantages of the PCR-sequencing combinations include: being expensive and time-consuming; requiring a moderately long, intact DNA; cannot be used to analyze mixed DNA samples; and not being suitable for highly degraded samples.

Instead of DNA sequencing, a real-time PCR assay for elephant species identification can provide both quantitative and qualitative information, with the added benefits of being highly sensitive and less prone to errors from post-PCR processes. Wozney and Wilson [14] developed a *TaqMan*-based real-time PCR assay for such purpose. Compared to *TaqMan* probes, the use of an intercalating dye in combination with melt curve analysis is cheaper and up to a few targets can still be multiplexed. Melt curve analysis has been widely applied for the identification of many species, subspecies, and even for body fluid analysis (e.g. [15–17]).

Real-time PCR with melt curve analysis has never been applied to elephant species identification. In this study, we aimed therefore to develop and validate the first fully functional real-time PCR assay based on the use of melt curve analysis of both Elephantidae- and elephant species-specific SNPs for a sensitive and accurate identification of the elephant species from which the ivory was taken. The assay developed in this study should be beneficial to wildlife forensic laboratories and those involved in combating wildlife crime.

2. Materials and methods

2.1. Sample collection

A total of 141 samples comprising 52 samples from Asian elephant (*Elephas maximus*), 62 samples from African bush elephant (*Loxodonta africana*), and 27 samples from African forest species (*Loxodonta cyclotis*) were collected from the DNP Wildlife Forensic Unit, Department of National Parks, Wildlife and Plant Conservation, Thailand and Songkhla Zoo, Thailand (Table 1). Samples were in the form of liquid blood or confiscated ivory. For liquid blood, species were confirmed by a veterinarian using gross morphological characteristics prior to drawing blood. These blood samples were collected by a veterinarian and kept in a vacutainer tube containing ethylenediaminetetraacetic acid (EDTA). To confirm the identity of the species from which the ivory samples came, PCR with universal *cyt b* primers followed by sequencing was performed prior to this study [18]. Ivory was incised from the inside of a proximal-hollow ivory called dentin then cut into small pieces, using a sterile scalpel and bone scissors. They were then kept separate in sterile plastic bags until further analysis.

Table 1
Details of elephant species, type of sample, sample number, and where they were collected from. DNP = DNP Wildlife Forensic Unit, Department of National Parks, Wildlife and Plant Conservation, Thailand. SZ = Songkhla Zoo, Thailand.

Elephant species	Sample type	Source	No. of samples
Elephas maximus	Confiscated ivory	DNP	2
	Blood	SZ	50
Loxodonta africana	Confiscated ivory	DNP	62
Loxodonta cyclotis	Confiscated ivory	DNP	27
Total			141

2.2. DNA extraction

Tiny pieces of ivory samples were placed in a 1.5 mL microcentrifuge tube until the tube was half-filled. The samples were decalcified using 700 μL of 0.5 M EDTA. The solution was left for seven days or until the sample softened before being scraped using a sterile scalpel. The scraped ivory sample was then transferred to a new 1.5 mL microcentrifuge tube and 20 μL of proteinase K (10 mg/mL) was added. The solution was incubated at 56 °C for 24 h and centrifuged at 11,000 rpm for 1 min. The supernatant was then used for DNA extraction using the Favorgen Stool Kit (Favorgen Biotech Corporation, Taiwan) while DNA from blood sample was extracted using the QIAamp DNA Mini Kit (Qiagen, UK), according to the 'Blood' protocol. The DNA extracts were stored at −20 °C until further analysis.

2.3. Direct PCR sample preparation

Direct amplification of elephant blood and ivory samples were adapted from Kitpipit et al. [19,20]. Tiny pieces of ivory samples were placed in a 1.5 mL microcentrifuge tube and soaked in 20 μL PBS for two min. The solution was then incubated at 98 °C for a further two min. From the PBS solution, 1.5 μL was added directly to the qPCR reaction mix.

2.4. Elephantidae- and elephant species-specific SNPs identification

To identify Elephantidae-specific SNPs, 10 sequences from each elephant species and 207 sequences from 147 mammalian species covering broad taxonomic groups were aligned using Mega 5 [21]. SNPs that were specific to Elephantidae and not present in other mammalian species were identified manually.

To identify elephant species-specific SNPs, all elephant cyt*b* and ND5 gene sequences available on GenBank at the time of the study were downloaded. A total number of 1403 sequences, comprising 483 from *E. maximus*, 779 from *L. africana*, and 141 from *L. cyclotis*, were obtained. These sequences were then aligned using the program Mega 5 [21]. Among these sequences, 14 (one from *E. maximus*, 11 from *L. africana*, and two from *L. cyclotis*) were found to contain several anomalies assumed to be DNA sequence errors and were removed from further analysis. The SNPs or nucleotide bases that were specific only to one elephant species were located manually.

2.5. Primer design

Elephantidae- and elephant species-specific primers were designed by designing the target SNPs to be at the last base of the 3′-end of the primers. The candidate primers were checked for their physical parameters such as annealing temperature (Ta), GC content, primer length, and secondary structures using the bioinformatics web-based tool Oligo Calc (http://www.basic.northwestern.edu/biotools/OligoCalc.html). Primers were ordered from Macrogen Inc., Korea. uMELT bioinformatics tool was used to predict and select for PCR products that have sufficiently different melting temperatures to enable unambiguous species identification [22]. Table 2 shows the primers used in the optimized assay.

2.6. Real-time PCR amplification

2.6.1. Conventional amplification

Singleplex and multiplex real-time amplification and melt curve analysis were performed using a CFX96™ real-time PCR

Table 2
Details of primers used in this study (primer name, strand (F/R for forward/reverse primer and H/L for heavy/light chain), primer sequence, and optimal primer concentration in a PCR). Both Emspec_cytb_3432R2.2 and E_cytb_870R.2 pair with Espec_cytb_870F.

Primer name	Strand	Primer sequence 5′→3′	[Opt] μM
LC_ND5_1681F1.1	F (H)	TGACTAGAAAAAATAATTCCCAAGAA	4.0
LCspec_ND5_1681R1	R (L)	CGGCTATTTTCTGCATAGTTATTAGA	4.0
Espec_cytb_870F	F (H)	CTTACGCCATTCTACGATCTGTA	0.5
E_cytb_870R.2	R (L)	GGACACCTCCTAGTTTGTTTGGT	0.3
Emspec_cytb_3432R2.2	R (L)	GGCCAATAATGGTGTAGGGG	0.2

system (Bio-Rad, CA, USA). The PCRs were performed in a total volume of 20 μL, comprising 10 μL SsoFast EvaGreen Supermix (containing Sso7d-fusion polymerase, dNTPs, and PCR buffer), five primers at the optimal concentrations (Table 2), and 1 μL DNA template (conventional protocol; equivalent to 0.1 ng to 100 ng of DNA template) or 1.5 μL pre-PCR solution (direct protocol). Amplification conditions were as follows: enzyme activation at 98 °C for 2 min; 35 cycles of denaturation at 98 °C for 5 s, annealing and extension at 60 °C for 5 s. Immediately after amplification, melt curve analysis was carried out as follows: 95 °C for 10 s, and stepwise annealing from 60 to 84.9 °C with 0.3 °C increments. Melt curves were converted into melting peaks by plotting the negative derivative of fluorescence by the derivative of temperature ($-d(Rfu)/dT$) against temperature (T). The melting peaks were called manually based on a noticeable difference from the no template control. Melting peaks were then used to determine the specific PCR products.

2.6.2. Nested amplification

For nested PCR, 0.2 μL PCR products from the first round of amplification were used as the template for amplification. All other reagents, including the primers, were the same as previously described.

2.6.3. Assay validation

The developed direct-multiplex assay was validated for its accuracy, sensitivity, and specificity. The assay's accuracy and its practicality for real forensic casework analysis were assessed using 141 ivory and blood samples from the three elephant species. Additionally 10 ivory products were included to determine the assay's performance with highly processed samples.

To assess the sensitivity of the assay (limit of detection), DNA extracts from voucher samples were quantified using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, USA). These DNA samples were used to prepare five DNA concentrations using a two-fold serial dilution, from 0.250 ng to 0.002 ng. They were then analyzed by the developed assay.

Thirty-two other mammalian species commonly found as wildlife forensic evidence or as domestic contamination were used to test the specificity of the assay. These species comprised tiger (*Panthera tigris*), lion (*Panthera leo*), leopard (*Panthera pardus*), white rhino (*Ceratotherium simum*), banteng (*Bos javanicus*), bison (*Bos gaurus*), buffalo (*Bubalus bubalis*), camel (*Camelus dromedarius*), eld's deer (*Panolia eldii*), hog deer (*Axis porcinus*), sambar deer (*Cervus unicolor*), wild boar (*Sus scrofa*), Malayan tapir (*Tapirus indicus*), Indian small civet (*Viverricula indica*), Asiatic brown bear (*Ursus thibetanus*), Malayan sun bear (*Ursus malayanus*), chimpanzee (*Pan troglodytes*), Southern pig-tailed macaque (*Macaca nemestrina*), orangutan (*Pongo pygmaeus*), dusky leaf monkey (*Trachypithecus obscurus*), common gibbon (*Hylobates lar*), Bengal slow loris (*Nycticebus bengalensis*), sheep (*Ovis aries*), rabbit (*Oryctolagus cuniculus*), horse (*Equus ferus caballus*), goat (*Capra hircus*), cow (*Bos taurus*), goose (*Anser domesticus*), duck (*Anas platyrhynchos*), domestic dog (*Canis lupus familiaris*), domestic cat (*Felis catus*), and human (*Homo sapiens*). The species of all samples were confirmed using gross morphological characteristics by a veterinarian and a museum curator prior to sample collection (i.e. blood or hair).

To further test the accuracy of the assay, single-blind testing was performed using 15 samples. For each elephant species, five were randomized from the original sample pool described in sample collection. These samples were then analyzed using the developed assay.

2.6.4. Evaluation of direct amplification and nested amplification

Twenty confiscated ivory samples and ten blood samples were randomly selected from the sample pool of 141 samples. These 30 samples were analyzed with the assay using both DNA extracts (conventional protocol) and the pre-PCR solutions (direct protocol). Additionally, 30 aged ivory samples (seized date between 1992 and 1996 with no species previously identified) that were not amplifiable or interpretable in a prior study using the PCR-sequencing protocol for species identification were analyzed with the developed assay. Both DNA extraction protocol and direct PCR protocol were used for these samples. Nested PCR was also employed as described in the earlier section to evaluate whether they are useful for these aged samples.

3. Results

3.1. Elephantidae- and elephant species-specific SNPs identification

Identification of Elephantidae-specific SNPs and elephant species-specific SNPs were performed manually. Two SNPs at position 15,182 and 13,431 were specific to *E. maximus* and *L. cyclotis*, respectively, in the 1389 sequences aligned. No *L. africana*-specific SNP was found in the entire cyt*b* and ND5 gene sequences downloaded. One Elephantidae-specific SNP was identified at position 14,999 using 207 mammalian sequences from 147 species. Table 3 shows the location of the SNPs and number of sequences in which the SNPs were observed.

3.2. The development of real-time PCR assay with melt curve analysis

DNA samples from the three elephant species were used for assay development. The developed assay generated three PCR products: an *L. cyclotis*-specific PCR product of 52 bp with a melting peak at about 68 °C, an *E. maximus*-specific product of 225 bp with a melting peak at about 79 °C, and an Elephantidae-specific product of 45 bp with a melting peak at about 72 °C (Fig. 1). An *L. cyclotis* sample showed two melting peaks – the *L. cyclotis* specific product and the Elephantidae-specific product. Similarly, an *E. maximus* sample generated both the *E. maximus* product's melting peak and the Elephantidae-specific product's melting peak. A single Elephantidae-specific product at 72 °C indicated an *L. africana* sample,

Table 3
Elephantidae- and elephant species-specific SNPs. The SNP positions are shown in parentheses in the heading. The numbers of sequences containing those SNPs are shown with their corresponding nucleotides based on 147 mammalian species.

Species	*E. maximus*-specific SNP (15,182)		*L. cyclotis*–specific SNP (13,431)		Elephantidae-specific SNP (14,999)	
E. maximus	C	254	C	201	A	10
L. africana	T	700	C	551	A	10
L. cyclotis	T	135	T	96	A	10
Other 147 mammals					C/T	99/37

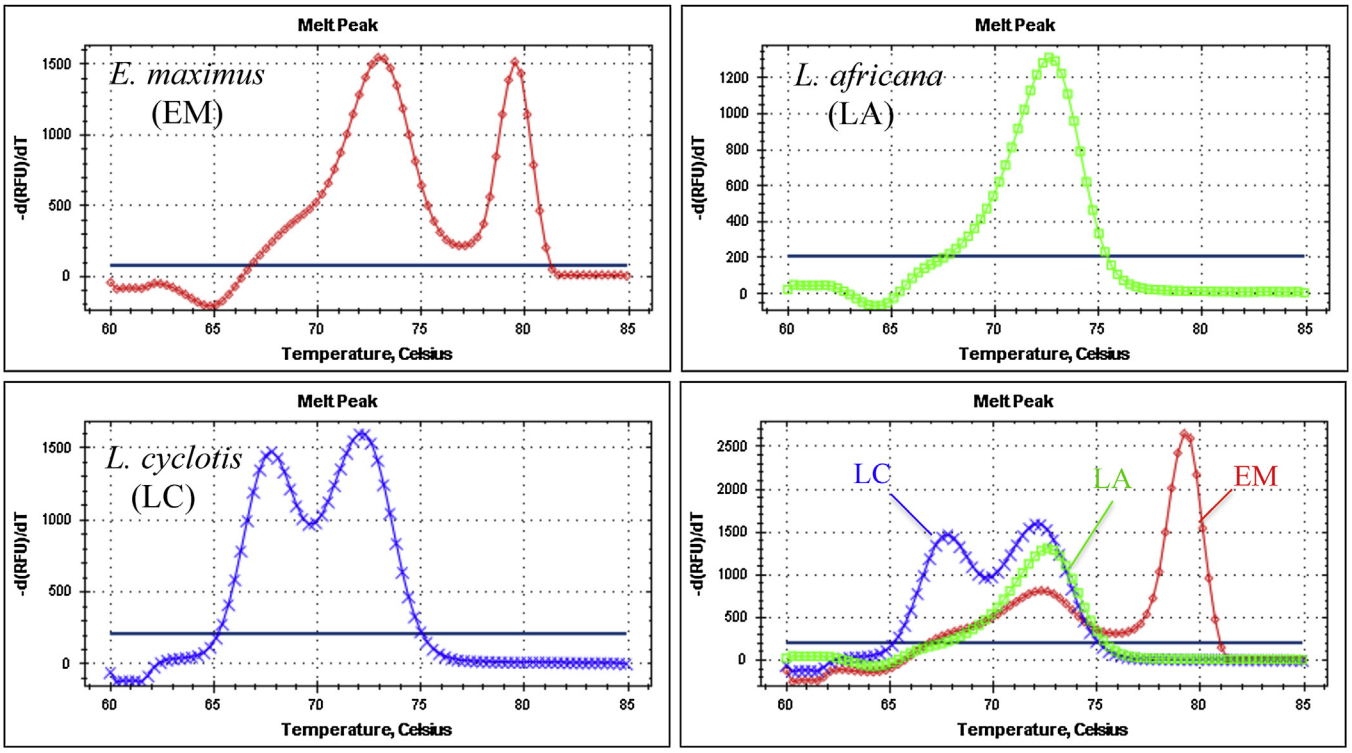

Fig. 1. Melting peaks of three elephant species displayed separately and together in one panel. In order of increasing melting temperatures, the melting peak at 68 °C is *E. maximus* specific, 72 °C is Elephantidae-specific, and 79 °C is *L. cyclotis* specific. *E. maximus* sample shows two peaks at 72 °C and 79 °C (red circle). *L. cyclotis* sample shows two peaks at 68 °C and 72 °C (blue cross). *L. africana* sample only shows the Elephantidae-specific peak at 72 °C (green square). One representation sample was used to generate each curve. (For interpretation of the references to color in this legend, the reader is referred to the web version of the article.)

as *L. cyclotis* and *E. maximus* would have generated their respective PCR products and corresponding melting peaks. All negative controls exhibited no increase in Evagreen fluorescence.

3.3. Reproducibility test

A total of 141 elephant DNA samples extracted from both blood and confiscated ivory were used to test the accuracy of the assay and its competency for forensic casework samples. Fig. 2 shows the melting peaks of all the samples, categorized by elephant species. 136 of 141 (96.5%) were correctly classified based on their melting peaks. No overlap in the melting peaks for the three different PCR products was seen ($p < 0.001$ for all pair-wise comparisons with Tukey's HSD). As expected, *E. maximus* samples had the lowest average C_q and standard deviation (18.13 ± 1.80), as the samples were mostly blood while the other samples from the other two species were confiscated ivory. Identification accuracy for Asian elephant was found to be 90.2% compared to that of 100% for the other two species.

3.4. Specificity test

All the 32 other mammalian species did not generate an increase in fluorescence signals during the 35 cycles of PCR (Fig. 3). This indicates that they were either not amplified or had very low amplification efficiency with the assay. Minimal increase was observed near the last cycles but they did not cross the software-determined threshold. Thus, all non-target DNA had C_qs as "undetermined". Small, insignificant melting peaks were observed for some samples but they were easily distinguished from the target PCR products, as the fluorescence signals of the non-targets were three to a 100 times lower than those of the targets. Moreover, the melting peaks of the non-target DNA samples that occurred near to the Elephantidae-specific peak at 72 °C were shifted by 0.5 °C to 1 °C to the left.

3.5. Sensitivity test

Five dilutions of the DNA template were used to determine the sensitivity of the assay (i.e. the minimum amount of each target elephant species DNA that could still be detected). The minimum amount of input DNA that could be amplified and detected was 0.063 ng, 0.031 ng, and 0.031 ng for *E. maximus*, *L. africana*, and *L. cyclotis*, respectively (Fig. 4). At lower input amounts, the peaks were noticeable but they were more difficult to distinguish from no template controls. With nested amplification, the minimum amount was 0.002 ng for all three species (Fig. 4).

3.6. Blind trial test

Random single-blind trial was performed with 15 Elephantidae samples to further test the accuracy of the assay in casework-like situations. Every sample was successfully amplified and their melting peaks were also successfully generated. Fig. 5 shows the melting peaks of the 15 blind samples and the predicted species. All samples were correctly classified, i.e. the assay displayed 100% accuracy with the blind samples.

3.7. Evaluation of direct amplification and application in aged samples

To evaluate the possibility of using direct PCR from elephant samples with the developed assay, 30 samples comprising confiscated ivories and blood samples were directly amplified by the assay. The result shows that blood ($n = 10$) and ivory ($n = 20$) had 100% (10/10) and 80% (16/20) success rates using the direct

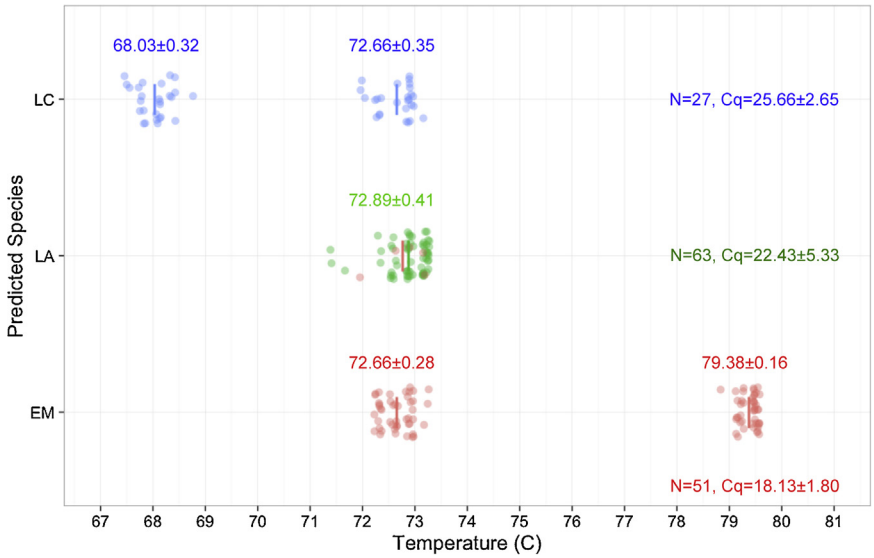

Fig. 2. Melting peak temperatures and their means (vertical bar) are shown for each elephant species (LC = *L. cyclotis*, LA = *L. africana*, and EM = *E. maximus*). Each circle represents one sample (shown with random jittering). The total numbers of samples for each species, average quantification cycles, and the standard deviations are also shown. No overlap is seen for the three PCR targets' melting peaks ($p < 0.001$). Five *E. maximus* samples were misidentified as *L. africana* (red dots in the LA category) due to the lack of the *E. maximus*-specific peaks at 79 °C.

real-time protocol, respectively (data not shown). Of the 16 successfully amplified ivory samples, 14 gave an interpretable result in the first attempt. The other two samples were amplifiable and provided the expected result when they were re-amplified. Four of the 30 ivory samples did not generate a PCR product in both the first and second analysis.

Thirty aged ivory samples that could not be analyzed by prior PCR-sequencing of the amplification product were analyzed with the developed real-time PCR assay. Both conventional extraction protocol and direct PCR protocol were used in combination with nested amplification. For non-nested amplifications, only three samples (out of 30) generated interpretable melting peaks

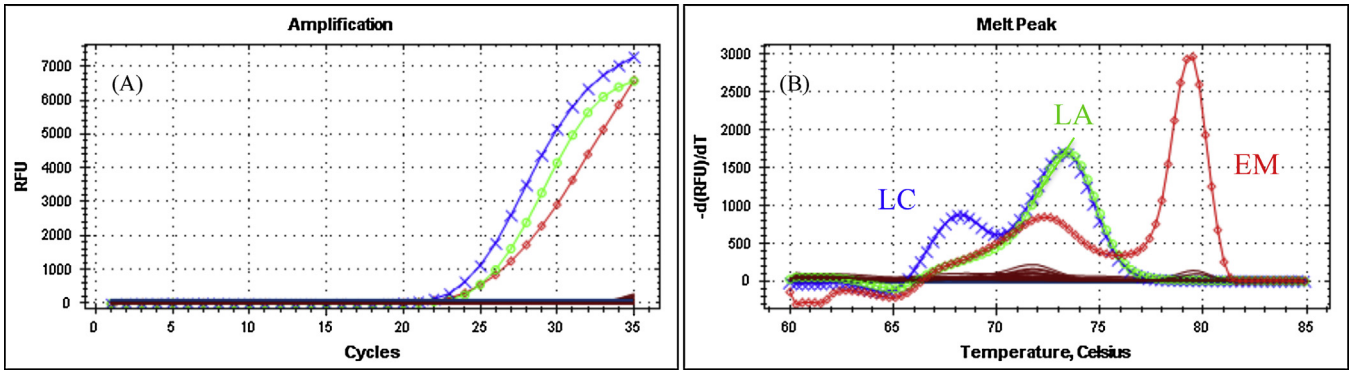

Fig. 3. Using DNA from 32 mammalian species, only elephant DNA were amplified and detected by the assay. All other species did not cross the threshold at 35 cycles (A). Small melting peaks were observed in some species but were extremely small compared to the peaks from elephant DNA samples (B).

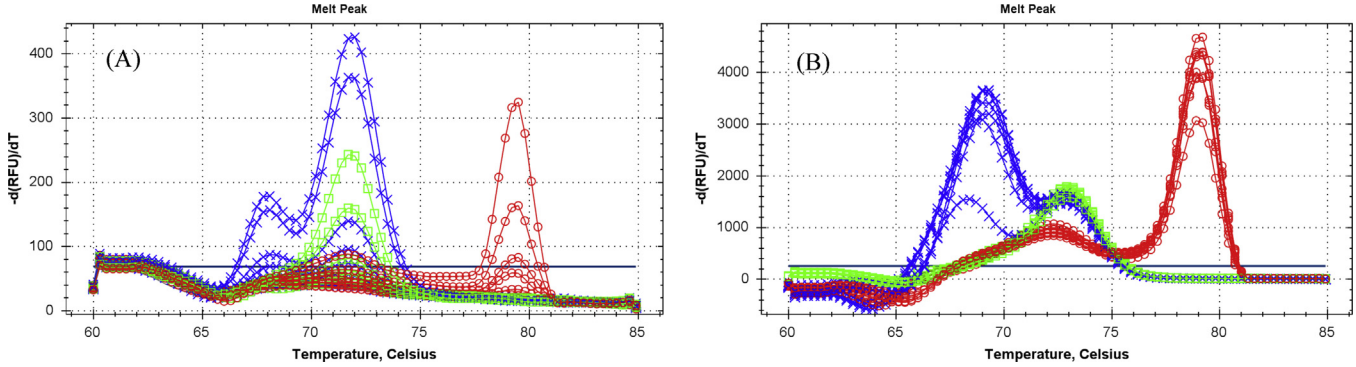

Fig. 4. Melting peaks from sensitivity test using (A) conventional amplification and (B) nested amplification. DNA concentrations ranged from 0.250 ng to 0.002 ng (*E. maximus* = red circle, *L. africana* = green square, and *L. cyclotis* = blue cross). (For interpretation of the references to color in this legend, the reader is referred to the web version of the article.)

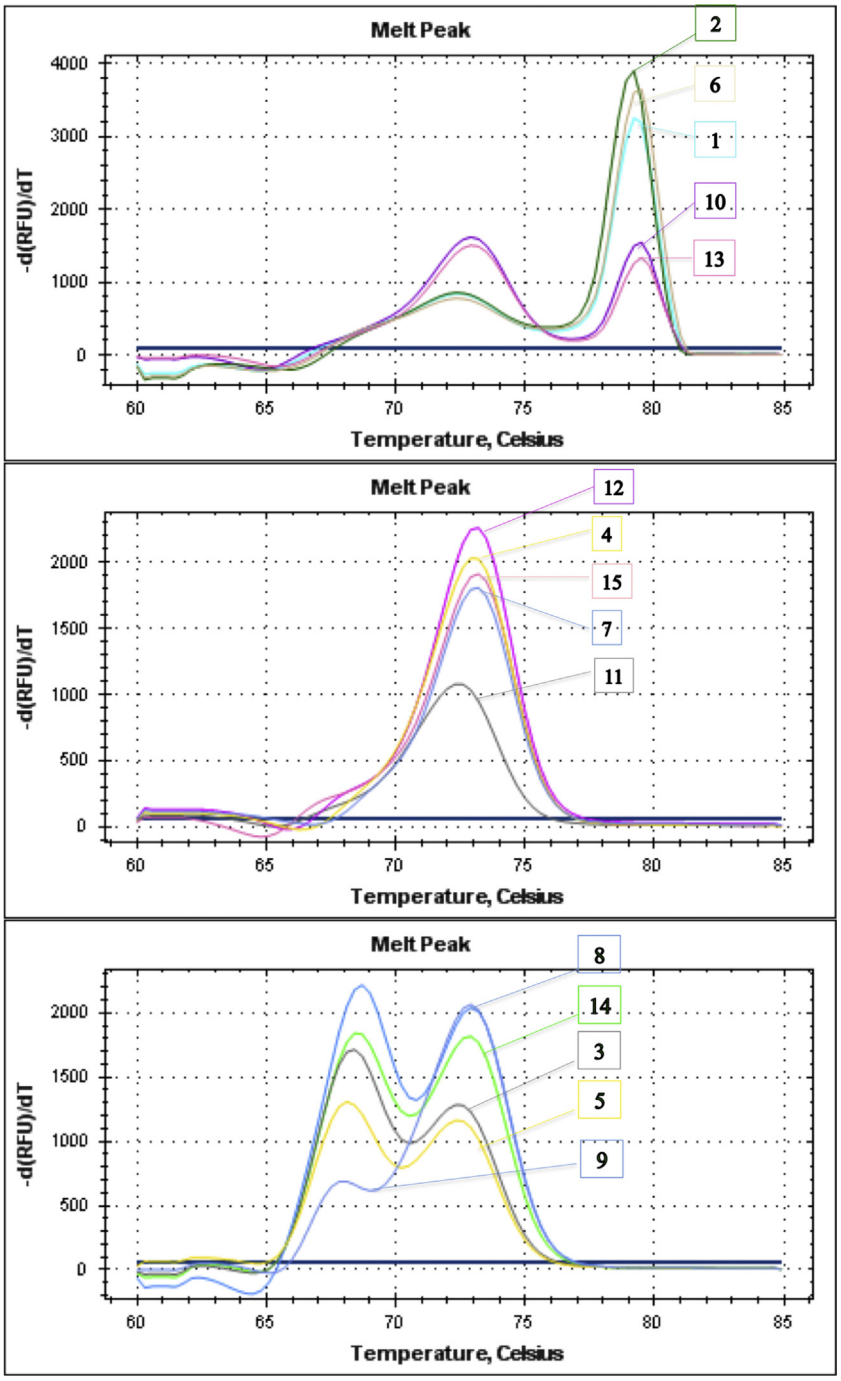

Fig. 5. Melting peaks obtained from the 15 single-blind samples. All samples were correctly classified based on their melting peaks (top = *E. maximus*, middle = *L. africana*, and bottom = *L. cyclotis*).

(Fig. 6 – red circles). Of the three, two were identified as *L. africana* with direct PCR, while conventional PCR failed to amplify the samples. Conversely, one sample was identified as *L. cyclotis* with conventional PCR while direct PCR failed.

When nested amplification was used, 24 of 30 samples were amplifiable with either conventional or direct PCR (Fig. 6 – green triangles). Twenty-one of these were concordant, and only three were discordant. Of these three discordant samples, two samples were subsequently identified as *L. africana* with direct PCR, while conventional PCR failed to amplify either of these two samples. The remaining one sample was identified as *L. cyclotis* with direct PCR and *L. africana* with conventional PCR.

4. Discussion

This is the first time that a multiplex real-time PCR assay has been successfully developed and fully validated for elephant species identification from blood, confiscated ivory, and aged ivory samples. The assay could reliably and accurately identify the three extant elephantid species using differences in melting temperatures. The non-overlapping peaks led to unambiguous species calls, and the absence of cross-reactivity shows that the assay could be used reliably to identify elephantids from other mammals. The assay could be completed in only 90 min due to its the capability of being used in a direct PCR format. It is cheaper, less cumbersome,

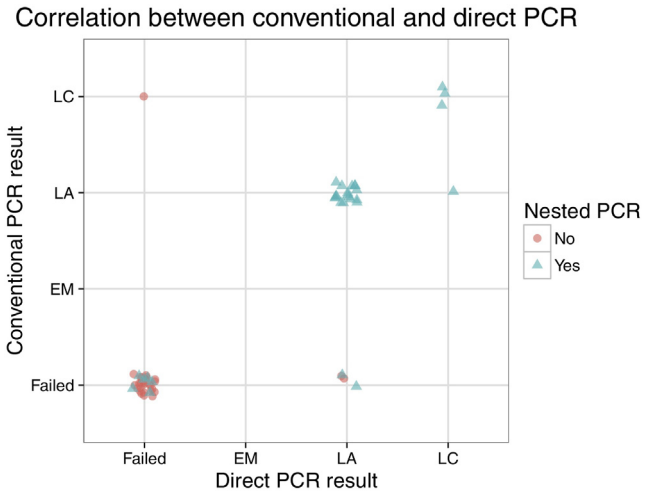

Fig. 6. Species identification result of 30 aged ivory samples using both conventional (*y*-axis) and direct PCR (*x*-axis) with nested amplification. Red circles indicate samples that underwent one round of PCR while green triangles indicate samples that underwent nested amplification. *x*- and *y*-axes show predicted species based on melting peak patterns. (For interpretation of the references to color in this legend and in the text, the reader is referred to the web version of the article.)

and more sensitive than conventional PCR-sequencing. Additionally, our real-time assay utilizes an intercalating dye in combination with well-designed primers, making it cheaper than a real-time assay with hydrolysis probes.

The SNPs identified in this study reinforce the usefulness of Genbank and BOLD for designing a species-specific assay. Non-regulated sequences in Genbank again cautions that submitted sequences must be rechecked before being used. As demonstrated by the number of sequences available, there is no shortage of elephantid DNA data, but more *L. cyclotis* sequences could possibly help to uncover *L. africana*-specific SNPs, as these two species are very closely related and hybrids are often found in nature ([23]). The transitions (C to T change) observed between *E. maximus* and *L. cyclotis* and the transversions (A to C/T change) between elephantids and other mammals were expected as transitions are more commonly observed in closely related species [24].

The melting peak approach has been used widely for species differentiation in forensic science and other related fields with great success (e.g. [15–17]). A PCR product's melting temperature depends on many factors, such as product length, sequence composition, secondary structure, and the salt environment [22]. Melting can happen abruptly for the whole length of a PCR product but dissociation can also happen in sections as the temperature increases. In this work, the PCR products were designed such that they separate as completely as possible at a single temperature so that the resulting melting peak is sharp and rapid. Another criterion was that the three products should have at least 4 °C difference in melting temperatures. Many candidate primer pairs and PCR products were evaluated using uMELT simulations and narrowed down to a few prior to the actual experiments. As such, unambiguous identification of the three extant elephant species could be achieved, as shown by the non-overlapping peaks in the 141 blood and confiscated ivory samples tested as well as in the 30 aged ivory samples.

Real-time PCR cuts down the analysis time by more than three hours compared to PCR-sequencing. It is also less labor-intensive and has much lower risks of contamination. Compared with the *Taqman*-based assay developed by Wozney and Wilson [14] to identify three elephantids (*L. africana, E. maximus*, and *Mammathus primigenius*), our Evagreen-based assay can distinguish between the two extant *Loxodonta* spp. This feature could help CITES and other relevant organizations to identify poaching hot-spots, as these two African elephant species inhabit different areas. Unlike the published *Taqman*-assay, this assay was designed to be qualitative only for two reasons. One, we wanted to investigate the possibility of using a direct PCR protocol to further drive down the assay cost and analysis time. Two, there is no need to run two to three lanes of DNA standards, which reduces the cost of the assay. The minimum analysis cost is currently less than 1 USD per sample, allowing use in forensic wildlife units in developing countries where ivory black markets thrive.

With the direct protocol, calcium from ivory could potentially inhibit the real-time PCR through competition with magnesium for polymerase cofactor sites [25]. The use of SsoFast polymerase with Sso-binding domain probably helps to amplify samples in the presence of calcium, as SSoFast has been shown to be more inhibitor tolerant than traditional *Taq* polymerase [26].

The use of PCR-sequencing with universal primers has the benefit that no prior sequence knowledge is required. However, they cannot be used to analyze a mixture of elephant ivory (e.g. composite talisman) or a contaminated ivory sample (e.g. *L. cyclotis* blood on *L. africana* ivory smuggled in the same shipping cargo). Also, our target sizes of 45, 52, and 225 bp are shorter than that used for a typical universal primer assay (e.g. 486 bp in Lee et al. [10] and 357 bp in Lee et al. [13]), which probably explain our high success rates. As such, the developed assay was able to amplify all blood samples and confiscated ivory samples (100% success rate with DNA extraction) without nested amplification. The use of only a single round of amplification for blood samples and samples typical of confiscated ivory minimizes the likelihood of contamination as no PCR product tube is opened for nested amplification. Lower success rates were seen with aged ivory samples, but this problem was circumvented with nested amplification (as in [10]), albeit with an increased risk of contamination. Because it was not known which species the aged ivory samples originated from and the possibility of amplifying contaminated DNA, it was not possible to say which result was correct. It was likely that the nested amplification protocol picked up low-level contaminating DNA on the ivory, as some of them were collectively burnt prior to being displayed in museums.

The limitations of the developed assay are (1) the misidentification of five *E. maximus* samples, (2) lack of quantitative information, (3) small, broad peaks from non-target species, and (4) the lack of close genetic relatives of the extant elephants for specificity test. First, five *E. maximus* samples were misidentified as *L. africana* due to the absence of the *E. maximus*-specific peak at 79 °C. The most probable reason for the missing high molecular weight PCR product (225 bp) was DNA degradation. All five samples displayed smearing on gel electrophoresis (data not shown). In practice, one could reduce the number of misidentified cases by checking the extracted DNA quality first, but this involves an additional step as well as wastes precious DNA extracts. Secondly, the developed test is not quantitative although this was deemed unnecessary, as the knowledge of DNA quantity is currently not necessary for the criminal justice process, but species assignment is required. Third, the slightly raised broad peaks seen with some non-targets was most likely due to low-efficiency amplification by the primers used in the assay. However, this did not interfere with interpretation of results and could not be confused with the peaks from extant elephant DNA samples. Last, mammoths, hyraxes, and dugongs – the three closest genetic relatives of elephants – were not included in the specificity test. However, bioinformatics check with NCBI BLAST showed that the primers could not bind to and amplify these species, except for Elephantidae-specific primers and mammoths. This is due to the fact that mammoths are also Elephantids and thus their mtDNA also contain the base A at SNP position 14,999 (data not shown).

The strength of this study lies in the large sample size used for the validation process, its specificity and sensitivity, and the low analysis time and cost required. Furthermore, this assay provides a diagnostic result based on differences in melting peaks, which eliminate ambiguities associated with the traditional PCR-sequencing protocol. Given sufficient DNA quality and the absence of cross-reactivity with other wildlife species from the same geographical area, domestic species that are commonly found, and other species whose products can be confused with ivory (such as rhino, buffalo, bovine horns), a confident elephant species call can be made using the developed assay.

5. Conclusion

In this study we developed a multiplex, real-time PCR assay for elephant species identification. The assay is based on an intercalating dye and identification is achieved through different melting peak patterns. With the potential of being used in a direct PCR format, a nested amplification format, and the complete validation carried out in this study, wildlife forensic laboratories have another valuable tool at their disposal to combat one of the modern poaching crises.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.forsciint.2016.08.037.

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